Venom gland extract is not required for successful parasitism in the polydnavirus-associated endoparasitoid *Hyposoter didymator* (Hym. Ichneumonidae) despite the presence of numerous novel and conserved venom proteins

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**Abstract**
The venom gland is a conserved organ in Hymenoptera that shows adaptations associated with life-style diversification. Few studies have investigated venom components and function in the highly diverse parasitic wasps and all suggest that the venom regulates host physiology. We explored the venom of the endoparasitoid *Hyposoter didymator* (Campopleginae), a species with an associated polydnavirus produced in the ovarian tissue. We investigated the effects of the *H. didymator* venom on two physiological traits of the host *Spodoptera frugiperda* (Noctuidae): encapsulation response and growth rate. We found that *H. didymator* venom had no significant effect on host cellular immunity or development, suggesting that it does not contribute to parasitism success. The host physiology seemed to be modified essentially by the ovarian fluid containing the symbiotic polydnaviruses. Proteomic analyses indicated that the *H. didymator* venom gland produces a large variety of proteins, consistent with the classical hymenopteran venom protein signature, including: reprolysin-like, dipeptidyl peptidase IV, hyaluronidase, arginine kinase or allergen proteins. The venom extracts also contained novel proteins, encoded by venom genes conserved in Campopleginae ichneumonids, and proteins with similarities to active molecules identified in other parasitoid species, such as calreticulin, reprolysin, superoxide dismutase and serpin. However, some of these proteins appear to be produced only in small amounts or to not be secreted. Possibly, in Campopleginae carrying polydnaviruses, the host-modifying activities of venom became redundant following the acquisition of polydnaviruses by the lineage.

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1. Introduction

The venom gland is a conserved organ in Hymenoptera, but questions about the evolution and functional diversity of venom proteins remain unanswered. The use of high-throughput technologies has made a large contribution to improving our knowledge of hymenoptera venom components in the last 10 years (Asgari and Rivers, 2011; Formesyn et al., 2012). However, research has long focused on Aculeates such as bees, social wasps, ants or solitary hunting wasps (dos Santos et al., 2011; Piek, 1986; Wood and Hoffman, 1983) whereas Terebrant lineages (parasitic wasps) have been less studied. Parasitic wasps are a group of particular interest for investigations of the evolution of the nature and function of Hymenoptera venoms due to the extreme diversity of their lifestyles. Studies of phylogeny suggest that they are descended from an ancestor with a phytophagus lifestyle (Quicke, 1997; Sharkey et al., 2012) and they include ectoparasites and endoparasites that could be idobiomts (no regulation of the host) or...
koinobions (active manipulation of the physiology of the host). The generally accepted model is that endoparasites evolved from ectoparasites (Gauld, 1988; Whitfield, 1998). However, recent studies suggest a more complex picture with species evolving back and forth between the ectoparasite and endoparasite lifestyles (Sharkey et al., 2012). In parallel with these transitions in lifestyles, parasitoid venom may have undergone changes in function. Venom in Hymenoptera thus switched from its ancestral function in phytophagous species of being a lubricant during egg laying to a role in parasitic wasps of modifying host physiology to facilitate successful parasitism (Asgari, 2012; Gauld et al., 1988; Piek, 1986).

Host manipulation by parasitoid venoms displays great diversity between parasitoid species with different lifestyles (Asgari, 2012). Induction of host parasitization, that may facilitate oviposition and larval feeding, is a frequent effect of ectoparasitoid venoms (Piek, 1986; Quistad et al., 1994; Yarnamoto et al., 2007). For example, a set of paralytic peptides has been described in the venom of Bracon hebetor, and these peptides also display insecticidal activity (Quistad et al., 1994). Endoparasitoid venoms may also induce transient parasitism; this is the case for the ichneumonids Pimpla hypochondriaca (Parkinson et al., 2002b) and Venturia concescens (Piek, 1986), and the braconids Asobara spp. (Mabiala-Mboumdong et al., 2010) and V. bunius injected by Saasion et al., 2010). Venoms can modify host development, for example delaying in molting, altering pupation or reducing weight gain (Richards and Edwards, 1999). Such effects have been described for ectoparasitoid species from the genus Euplectrus (Nakamatsu and Tanaka, 2003) and the endoparasitoid Peromatus puparum, whose venomous proteins may disturb the host endocrine system (Zhu et al., 2009). However, only a few venom molecules acting on host development have been characterized. They include a represilin-type metalloprotease from Eulophus pennicornis venom that induces developmental alterations in the host Lecanobia orleacea (Price et al., 2009), and a venom gamma-glutamyl transpeptidase of the braconid Aphidius ervi involved in aphid host castration by inducing apoptosis in the host ovarioles (Falabella et al., 2007). Venom components can alter host immunity. Indeed, the presence of large foreign objects such as parasitoid eggs inside the insect body triggers an immune response that leads to their encapsulation and death (Strand, 2012). This phenomenon generally involves the recruitment and adhesion of hemocytes that form a cell multilayer around the foreign invader (Aylin et al., 2011), as well as activation of the phenoloxidase (PO) cascade that leads to melanization of the capsule and production of toxic radicals (Cerienis and Söderhäll, 2004). Diverse venom proteins including the hemocyte anti-aggregation protein Vrp3 from P. hypochondriaca (Richards and Dani, 2008), the calrepticin from the braconid Cotesia rubecula (Zhang et al., 2006), and a RhoGAP from the figidit Leptopilina boulardi (Labrosse et al., 2005) have been shown to inhibit the cellular immune response. Venom proteins that alter the melanization response include serine protease homologs and a cysteine-rich/Kunitz-type protease inhibitor from C. rubecula (Asgari et al., 2003a, 2003b), an extracellular superoxide dismutase (SOD) and a serpin (LbSPhy) from L. boulardi (Colinet et al., 2011, 2009).

In numerous species, venom is the main source of factors regulating the host. However, symbiotic viruses i.e. polydnaviruses (PDV), virus like particles (VLP), and teratocytes are also sources of such factors (Beckage and Drezen, 2012). Braconids and ichneumonids can harbor PDVs or VLPs that are both produced in a specific ovarian tissue, the calyx, whereas figidit VLPs are produced in the venom apparatus. PDVs and VLPs are injected into the host during oviposition whereas teratocytes, large cells from the serosal membrane found in Braconids and Scelionids, are released into the host upon parasitoid hatching. These different factors from diverse sources may have synergistic effects on host regulation as such effects would confer a selective advantage on parasitoid species and therefore favor their emergence and maintenance through evolution. Complementarity between venom and other factors has indeed been demonstrated in a few models. For instance, venom from the braconids of the Cotesia genus (C. glomerata, C. kariyai and C. rubecula) and C. inanitus is required for successful parasitism and synergizes with the effects of the associated PDV (Asgari, 2012; Kaeslin et al., 2010; Kitano, 1986; Wago and Tanaka, 1989; Zhang et al., 2004). Also, a peptide in C. rubecula venom is involved in regulation of PDV gene expression in the lepidopteran host (Zhang et al., 2004). The venom of braconid wasps carrying PDV has thus retained functions in host regulation, at least as a co-factor for the associated bracovirus. Ichneumonid species are associated with symbiotic viruses from another PDV genus, the ichnoviruses (IV), whose ancestor is different to that of the bracoviruses (Volkoff et al., 2010). The venom glands of ichneumonid wasps appear to not necessarily produce host regulation factors: Campeoleis sonorensis venom is not needed for parasitism success, whereas the calyx fluid containing the PDVs is essential (Vinson and Stoltz, 1986; Webb and Luckhart, 1994), and Transoema rastrale venom is not involved in modifying host development (Doucet and Cusson, 1996). However, except for these few studies, there has been no rigorous systematic analysis of the contents and physiological effects of the venoms of ichneumonid species with associated PDVs.

We report the first exhaustive proteomic analysis of the content of the venom of an ichneumonid wasp carrying PDV: the Campopleginae Hyposoter didymator, a solitary koinobiont endoparasitoid of Noctuid larvae, associated with the Hyposoter didymator Ichnovirus (Hdv). We also analyzed the host (Spodoptera frugiperda) life traits following injection of venom, with or without calyx fluid. H. didymator venom did not affect host cellular immunity or development, or the number of parasitoids that succeeded in developing. This finding was surprising as proteomic analysis revealed a large set of proteins in H. didymator venom; they include both proteins previously described in other parasitic wasp venom, some involved in parasitism success, and proteins of unknown function encoded by genes strongly transcribed in the venom gland. Possibly, in ichneumonids carrying PDVs, the host-modifying activities of venom became redundant following the acquisition of viruses by the lineage.

2. Materials and methods

2.1. Biological materials

2.1.1. Insect rearing and parasitism

S. frugiperda was obtained from a laboratory strain and maintained at 24 ± 2°C, 65% relative humidity, and 16/8 light/dark on a semi-synthetic diet. H. didymator was maintained on S. frugiperda larvae in the same abiotic conditions, using 2nd instar larvae for parasitism. For experiments requiring 100% parasitism, S. frugiperda larvae (2nd, 3rd or 4th instar) were individually introduced into a glass vial containing 10 2-day-old H. didymator female wasps. The host larvae were removed immediately after being stung and maintained in an incubator as described above.

2.1.2. Preparation of H. didymator maternal extracts

Vernon and calyx fluid (containing the PDV particles) were extracted from H. didymator females anesthetized on ice and dissected in phosphate-buffered saline (PBS) under a light microscope. The venom apparatus (gland and reservoir) and the ovaries were collected separately and pooled in 250 μL PCR tubes. The volume was adjusted with PBS to reach the desired concentration in wasp equivalents (w.e., for example: venom apparatus from 10 wasps in
2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH = 7.4 for overnight at +4 °C then post-fixed with 2% (v/v) osmium tetroxide in the same buffer for 1 h at room temperature. Samples were dehydrated through an ethanol series and embedded in Epon. Ultrathin sections contrasted with uranyl acetate and lead citrate were examined under an electron microscope Zeiss EM 10 CR at 80 kV.

2.1.4. Injections in S. frugiperda larvae

Injections were given to S. frugiperda larvae anesthetized with CO2 using the Nanoject II™ Auto-Nanoliter Injector (Drummond). In all experiments, 100 nl of PBS (control), or 0.04 w.e. of venom, calyx fluid or a mixture of venom and calyx fluid in 100 nl were injected into 3rd or 4th instar S. frugiperda larvae, less than 1 day old. The S. frugiperda larvae were then kept individually in 24-wells plates at 25 ± 2 °C and fed with a semi-synthetic diet.

2.1.5. Isolation of H. didymator eggs for injection

S. frugiperda larvae (2nd or 3rd instar) were parasitized 2 or 3 times and rapidly dissected in PBS to recover the H. didymator eggs (2–3 eggs per larva). The eggs were pooled in a Petri dish containing 20 ml of PBS, washed twice with 20 ml of PBS, and stored in PBS at room temperature for less than 2 h before being injected into S. frugiperda larvae. The cleaned eggs were screened by TEM to ensure that the egg chorion did not contain HdIV particles (data not shown). Similarly, 3rd instar S. frugiperda larvae were each injected with two cleaned eggs. After 24 h, ten S. frugiperda larvae were collected to test for HdIV gene expression by RT-PCR (using primers specific to two genes known to be abundantly transcribed, M24 and P30) to further ensure absence of HdIV contamination (data not shown).

2.2. Effect of H. didymator venom and calyx fluid

2.2.1. Analysis of the parasitoid larval survival

To analyze the effect of H. didymator extracts on parasitoid larval survival, two 2 h-old H. didymator eggs were injected together with 100 nl of PBS or female wasp extracts into 4th instar S. frugiperda larvae. As a control, single parasitized S. frugiperda larvae were injected with 100 nl of PBS containing 1 egg. The number of parasitoid larvae was verified 10 days after parasitism, i.e. 2 days after the normal time at which mature larva egresses from the host. In the absence of H. didymator mature larvae, host caterpillars displaying symptoms of parasitism (instar delay and low weight) were dissected to determine the parasitoid status (dead or alive, egg or larva). In every case, only one alive parasitoid larva was observed in each dissected S. frugiperda host. Parasitoid larva survival (L_10d) was estimated as the number of parasitoids still alive as a percentage of the number of S. frugiperda larvae injected. Both the larvae still inside the host and those that had egressed from the host were counted. Egressed larvae were kept until day 19 to determine the proportion that reached adulthood (Ad_19d), also calculated as a percentage of the number of host caterpillars injected. A total of 116 S. frugiperda larvae were injected with PBS, 87 with venom, 135 with calyx fluid, and 116 with both calyx fluid and venom. As controls, 37 larvae were parasitized.

2.2.2. Measurement of bead encapsulation rate

To analyze the effect of venom and calyx fluid on the ability of S. frugiperda to encapsulate foreign bodies, 3 to 4 Sephadex G-75 beads (40–120 μm) were injected together with 100 nl of PBS or H. didymator extracts into S. frugiperda 4th instar larvae. As a control, single parasitized 4th instar S. frugiperda larvae were injected with 100 nl of PBS containing 3–4 beads. S. frugiperda larvae were dissected 12 h or 72 h post-injection (p.i.) in PBS to recover the beads. Recovered beads were photographed under a phase contrast microscope. The number of encapsulated beads was recorded for each condition and time. For each encapsulated bead, the sizes of the bead (B) and the hemocyte layer (H) were measured using Image J software. The bead covering factor (CovF) was calculated as: CovF = H/B. A total of 69, 46, 29, 39 and 46 beads were analyzed 12 h p.i. and a total of 122, 85, 69, 31 and 34 beads were observed 72 h p.i. for PBS, venom, calyx fluid, combined calyx fluid and venom injections, and parasitism, respectively.

2.2.3. Measurement of S. frugiperda larvae weight

To analyze the effect of venom and calyx fluid on the weight gain of S. frugiperda 3rd instar larvae, hosts injected with PBS (control) or with H. didymator maternal extracts were individually weighed 6, 24, 48 h, 6 and 7 days p.i. A total of 75, 151, 55, 36 and 42 larvae were weighed for PBS, venom, calyx fluid, combined calyx fluid and venom injections, and parasitism, respectively.

2.2.4. Statistical analyses

Both S. frugiperda 3rd instar weight gain and bead covering factors could be considered to be normally distributed. Therefore, ANOVA was used to compare average values obtained with different H. didymator maternal extracts. For S. frugiperda 3rd instar weight gain, one-way ANOVA was used to analyze venom, calyx fluid, combined calyx fluid and venom, and parasitism against PBS condition for the different post-injection times. For bead covering factors, a one-way ANOVA with interaction was used to test the effect of the conditions. For bead encapsulation rates and parasitoid survival, however, logistic regression with a generalized linear model specially designed for modeling binomial data using the logistic link function was used to compare average values (McCullagh and Nelder, 1989). For the effect of the conditions on bead encapsulation rates, differences between 12 h and 72 h p.i. times and the interaction between these two main effects were tested. The effects of the conditions on parasitoid survival rate (L_10d and Ad_19d) were tested. SAS software (SAS Institute Inc., 1999) was used for all computations.

2.3. Venom protein analyses

2.3.1. H. didymator reference transcriptome

Two H. didymator reference libraries were used for proteomic analyses. The first was a venom EST library. Total RNA was extracted from venom organs collected from adult wasps using the Qiagen RNeasy Mini Kit according to the manufacturer’s protocol. The cDNA library was then obtained using the Creator SMART cDNA Library Construction Kit (Clontech) according to the manufacturer’s protocol. A total of 3367 clones were sequenced by the Genoscope using BigDye Termination kits on Applied Biosystems 3730xl DNA Analyzers. After cleaning to remove vector stretches and polyA tails, and
elimination of sequences shorter than 100 nt, 2994 EST sequences were obtained. These ESTs were then clustered using the TIGR software TGICL Clustering tool (TGICL) (Negre et al., 2006); the 330 clusters identified constituted the first transcriptome database, DAT_Hd-EST, used for venom protein identification (see below). The second library was generated from total RNA extracted from eggs, larvae and adults using the Qiagen RNeasy Mini Kit according to the manufacturer’s recommendations. This RNA was used to produce an equilibrated cDNA library, and sequencing was performed using GS FLX (Roche/454), Titanium chemistry (performed by GATC Biotech AG, Germany). The 451,267 sequences obtained were cleaned and clustered (GATC Biotech AG). The final database, named DAT_Hd-454, contained a total of 130,275 clusters.

All the sequences for both libraries were then submitted to TGICL for assembly using CAP3 Assembly software (Pertea et al., 2003). The resulting database (DAT_Hd-Contig) contained a total of 19,416 contig sequences. Despite the risk of chimeras, this approach was expected to maximize the total length of the available sequences and the probability of obtaining sequences of S’ regions to facilitate peptide matching and similarity searches with other protein-coding sequences.

2.3.2. SDS-PAGE and protein identification

Venom proteins were extracted from the whole venom gland as described above (section 2.1.2). The proteins were separated using Precast 15% READY GEL (BIORAD), under denaturing conditions. Samples equivalent to the contents of 10 venom reservoirs were loaded in each lane. Electrophoresis was performed in 25 mM Tris—HCl pH8.8, 195 mM glycine, and 0.1% (w/v) SDS at a constant current of 35 mA. Gels were stained with colloidal blue (Fermentas), and gel slices were cut out. Enzymatic in-gel digestion was performed according to the modified Shevchenko protocol (Shevchenko et al., 1996). Briefly, gel slices were destained by three washes in 50% acetonitrile, 50 mM NH4HCO3 and incubated overnight at 25 °C (with shaking) with 15 ng/ml trypsin (Gold, Promega, Charbonnières, France) in 100 mM NH4HCO3. Tryptic fragments were extracted with 50% acetonitrile and 5% formic acid, and dehydrated in a vacuum centrifuge. Samples (1 μl) were analyzed on-line in a nanoESI LTQ_XL Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an Ultimate 3000 HPLC (Dionex, Amsterdam, Netherlands). Samples were desalted and pre-concentrated on-line on a Pepmap® precolumn (0.3 mm × 10 mm). They were eluted from the capillary (0.075 mm × 150 mm) reverse-phase column (Pepmap®, Dionex) with a gradient of 0–40% B in 60 min, 80% B for 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 300 nl/min. Nano-ESI was performed with a spray voltage of 2.4 kV, a heated capillary temperature of 200 °C, and a tube lens voltage of 140 V. A cycle of one full-scan mass spectrum (400–1600 m/z) at a resolution of 30,000, followed by five data-dependent MS/MS spectra was repeated continuously throughout the nanoLC separation. All MS/MS spectra were recorded using normalized collision energy (35%, activation Q 0.25 and activation time 30 ms), and an isolation window of 3 m/z. Data were acquired using Xcalibur software (v 2.0.7, Thermo Fisher Scientific, Waltham, MA).

Proteins were identified by searching against the entries in the three databases described above (DAT_Hd-EST, DAT_Hd-454 and DAT_Hd-Contig) with the Mascot v2.2 algorithm (Matrix Science Inc., Boston, USA), ProteomeDiscoverer v1.1 (Thermo Fisher Scientific, Waltham, MA) was used for data submission. Peptides with scores greater than the identity score (p < 0.05) were considered as significant matches. All spectra were manually validated for proteins identified. Considering only sequences where at least two peptides match, we identified 943 non-redundant sequences (927 assembled sequences from DAT_Hd-Contig, 15 singletons from DAT_Hd-454 and 1 singleton from DAT_Hd-EST) coding for proteins in the H. didymator venom extract.

2.3.3. Sequence analyses

All 943 sequences identified were automatically annotated by Blast similarity searches using the Blast2GO online tool (http://www.blast2go.com) (Conesa et al., 2005). The Blastx algorithm was used with a local non-redundant protein database (NCBI, release March 1, 2011) with an E-value > e−05 as the cut-off (Supplementary data 1).

The sequences were then assigned by manual annotation to the 22 functional categories usually found in transcriptomic and proteomic analyses of venoms (Supplementary data 2): hydrolases (peptidases, esterases, glycosylases and other hydrolases); enzyme inhibitors; oxidoreductases; transferases; isomerases; ligases; lyases; chaperones; substrate-specific transport proteins; storage proteins; mitochondrial proteins; nucleotide-binding proteins; proteasome complex proteins; ribosomal proteins; structural proteins; proteins involved in cell traffic; proteins involved in regulation of transcription/translation; proteins with other functions and finally, proteins with unknown function.

Sequence alignments were performed using the Multalin online tool (Corpet, 1988) and visualized by Jalview (Waterhouse et al., 2009). Peptide signal cleavage sites were predicted using SignalP4 (www.cbs.dtu.dk/services/SignalP).

2.4. Characterization of Hd-VenA and Hd-VenB venom genes

For each gene, available EST sequences were aligned using Codon code aligner (Codon Code Corporation) to generate a full-length consensus sequence. For amplification of corresponding cDNA or genomic sequences, specific PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu). The primers were located at the extreme ends, and when possible in the 5’ and 3’-UTR sequences, of the available consensus mRNA sequences. Sequences of the primers are given in Supplementary data 3.

H. didymator genomic DNA was extracted from pooled males using Wizard Genomics DNA Purification Kits (Promega) according to the manufacturer’s recommendations. Total RNA was extracted from ovaries, venom apparatus and head and thorax tissues dissected from 15 H. didymator females using RNeasy Mini kits (Qiagen) according to the manufacturer’s recommendations. DNA was eliminated by treatment of 10 μg RNA with 2 U of TURBO DNase (Ambion) in a total volume of 20 μl. Then, cDNA was synthesized from 5 μg RNA in a total volume of 20 μl using 200 U of SuperScript III (Invitrogen), 40 U of RNasin (Promega), 500 mM oligo-dT and 10 mM of each dNTP.

PCR was performed in a total volume of 50 μl using 1.25 U of GoTag DNA Polymerase (Promega) per reaction in a GeneAmp PCR System 2700 (Applied Biosystems). Each reaction contained 50 ng of genomic DNA or cDNA, 200 μM dNTP mix and 0.4 μM of each primer. Following an initial step at 95 °C for 5 min, the reaction mixtures were subjected to 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 59 °C for 30 s, and DNA extension for 2 min at 72 °C. The reaction was completed by a final extension step at 72 °C for 5 min.

Amplification products were purified using the MiniElute gel extraction kit (Qiagen) according to the manufacturer’s recommendations. Purified PCR products were ligated into pGEM-T Easy Vector System (Promega) and the resulting constructs were used to transform TOP10 electro-competent cells (Invitrogen). Plasmids were then purified with QIAprep Spin Miniprep kits (Qiagen) according to the manufacturer’s instructions.
Insert DNA sequencing was carried out by GATC using the PCR primers. Intron sequences were identified by alignment of genomic sequences with EST sequences using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2). (Larkin et al., 2007).

3. Results and discussion

3.1. Structure of the H. didymator venom apparatus

The venom apparatus of H. didymator (Fig. 1A) shows the typical tripartite organization of Hymenoptera (Piek, 1986; Quicke, 1997), with a venom gland connected to a large, transparent and bulb shaped venom reservoir, and a venom duct linking the reservoir to the ovipositor. The venom gland has a branched tubular structure as in other Campopleginae species such as C. sonorensis (Ferrarese et al., 2009), Venturia canescens and Bathyplectes curculionis (Ravallec et al., unpublished data).

Transmission electron microscopy (TEM) revealed that the gland is mainly constituted of large cells with few organelles but numerous secretory vacuoles (Fig. 1B). All cells contain intracellular canals, into which secretion products are released, probably by a fusion mechanism (Piek, 1986). Intracellular canals have been observed in venom glands of many parasitoid species and have been called end apparatus (Zhu et al., 2008) and vesicular organelles (Wan et al., 2006). As described in Leptopilina spp. (Ferrarese et al., 2009), a portion named the rough canal, bordered with numerous microvilli (detail in Fig. 1C), extended as far as a second portion, named the smooth canal, lined with cuticle. This supra-cellular system of canals in the gland allows secretory products to flow to the cuticle-lined gland lumen bordered by a second type of small and narrow cell forming the intimal layer, and then to the reservoir.

There are secretory cells close to the junction between the gland and the reservoir, but further downstream they are replaced by epithelial non-secretory cells, as described in other parasitoid species (Wan et al., 2006). These cells contain very few organelles and resemble squamous cells described in other parasitoid species (Wan et al., 2006) that have a role in the maintenance of the chitin intima of varying thickness that covers the lumen intima (Fig. 1D and E). Putative aggregates of venom components are found within the reservoir lumen or covering the intima (Fig. 1D). The venom apparatus is surrounded by a thin muscular sheath (detail in Fig. 1E), similar to the “type 2” venom apparatus (Edson and Vinson, 1979) described in braconid wasps (Asgari, 2012).

The last part of the venomous organ is the primary duct (Fig. 1A) that allows venom transport to the ovipositor. The duct presents a spiraled chitin structure, which probably prevents it from collapsing.

Fig. 1. Structure of H. didymator venom apparatus. A. Light microscopy overview of the venom apparatus of 2 day-old female H. didymator: venom gland (VG), venom reservoir (VR) and venom duct (VD) which opens into the ovipositor (OP). B. Venom gland observed by transmission electronic microscopy (TEM) showing ultra-structure of secretory cells (delineated by dashed lines): nuclei (Nu), secretory vesicles (SV), rough canals bordered by microvilli (RC), smooth canals lined with cuticle (SC), and lumen lined with cuticle (L) and bordered by cells of the intimal layer (IL). C. Details of the end of a rough canal bordered by abundant microvilli (mv), into which secreted products are discharged. D. TEM view of the venom reservoir showing the nuclei (Nu) of epithelial squamous cell (Sq), the uneven chitinous ridge (CR) lining the reservoir wall, and putative venom aggregates (VA) in the reservoir lumen (RL). E. Portion of the venom reservoir surrounded by muscle fibers (Mu).
3.2. Impact of venom extracts on parasitoid survival and host physiology

To assess involvement of *H. didymator* venom in parasitism success, we investigated the effect of venom injections (i) on parasitoid survival, (ii) on the host cellular immune response and (iii) on *S. frugiperda* larval development.

3.2.1. Parasitoid survival

Parasitoid eggs were injected with 0.04 w.e. of venom, calyx fluid, or both, into *S. frugiperda* 4th instar larvae, and parasitoid larval survival (L₁₀d) was calculated as the number of larvae recovered (inside or egressed from the host) as a percentage of the number of *S. frugiperda* injected (Fig. 2). Almost all parasitized *S. frugiperda* ensured survival of one parasitoid 10 days p.i.: survival was 95%. Survival was significantly lower (31%) in PBS-injected caterpillars, but similar (91%) in eggs injected with calyx fluid. Survival in eggs injected with venom (26%) was similar to that in eggs injected with PBS, and co-injection of venom with calyx fluid did not significantly change parasitoid survival compared to injection of calyx fluid only (venom plus calyx fluid: 84% vs. calyx fluid only: 91% survival). Thus venom extract, unlike calyx fluid, had no noticeable effect on parasitoid egg and larval survival and did not appear to complement the effects of maternal ovarian factors. However, in about 20% of the hosts, eggs were able to evade the *S. frugiperda* immune response and to develop in the absence of calyx fluid. This suggests either that they were injected in immune-deficient caterpillars or that PDVs are not the unique factors essential for successful development of *H. didymator* in this host. The numbers of parasitoid larvae that spun cocoons and of adults were proportional to the number of larvae that survived: injection of eggs with PBS or venom alone resulted in very few adults (0.8 and 2% of the hosts, respectively) whereas adults were obtained from 30% to 13% of the *S. frugiperda* hosts into which calyx fluid was introduced, whether by parasitism or injection (Fig. 2). However, only 4% of hosts co-injected with calyx fluid and venom lead to adult parasitoids, a value not significantly different from that for the PBS group. Thus the proportion of larvae that reached adulthood was much lower for the group with venom and calyx fluid co-injection than for the group with calyx fluid alone; possibly venom has a negative effect during *H. didymator* preimaginal development. This finding requires further investigation. Interestingly, similar observations have been reported for natural parasitism by *C. sonorensis*: ablation of the venom apparatus resulted in 13% more offspring than from control females (Webb and Luckhart, 1994).

*H. didymator* venom extract clearly did not improve larval survival in *S. frugiperda*. This does not imply that the host caterpillar is not affected by the venom. Therefore, we investigated host life history traits classically affected by endoparasitic wasp venoms, such as the immune response and development.

3.2.2. *S. frugiperda* cellular immune response

Encapsulation by *S. frugiperda* was evaluated by recovering Sephadex beads 12 h and 72 h after injection into 4th instar larvae and measuring both a quantitative (percent of encapsulated beads; Fig. 3A) and a qualitative (bead covering factor, covF; Fig. 3B) indicator. As expected, almost all beads recovered from PBS-injected *S. frugiperda* larvae were encapsulated (98% and 94% at 12 h and 72 h p.i. respectively; Fig. 3A) and covered with a thick layer of hemocytes (covF = 1.2; Fig. 3B and C). For parasitized larvae, the percentage of encapsulated beads was significantly lower (62% and 75% at 12 h and 72 h p.i., respectively; Fig. 3A) and beads were only partially encapsulated or covered with a thin layer of hemocytes (covF = 0.5; Fig. 3B and C). Parasitism thus inhibited the *S. frugiperda* immune response. To investigate the involvement of venom in this phenomenon, beads were co-injected with 0.04 w.e. of venom, calyx fluid, or both. Injections of venom led to results similar to PBS injection, with up to 92% of the beads substantially encapsulated (covF = 1) at all times p.i. tested (Fig. 3A and B). After injection of calyx fluid, the number of encapsulated beads was initially much smaller (10% at 12 h p.i.); this value was much lower than that in parasitized larvae 12 h p.p., probably because the dose of calyx fluid injected may be higher than that females normally inject during parasitism. The percentage of encapsulated beads increased to 69% 72 h p.i., reaching a value and a coverage factor (covF = 0.45) similar to that observed in parasitized larvae (Fig. 3A and B). Addition of venom to the calyx fluid did not significantly change the results (Fig. 3B and C). These experiments indicate that the alteration of encapsulation observed in parasitized larvae is entirely due to calyx fluid factors with venom components making no significant contribution.

3.2.3. *S. frugiperda* larval growth

We investigated the effects of the venom on the development of *S. frugiperda* larvae by measuring the weight gain of parasitized and of injected caterpillars. Newly molted 3rd instar *S. frugiperda* larvae were weighed at various times after parasitism or injections of PBS or 0.04 w.e. of calyx fluid and/or venom extracts. Compared to PBS-treated larvae, parasitism significantly reduced larval weight gain from 24 h post treatment until the 7th day, one day before egression of the mature parasitoid larva (Table 1). Injections of venom extract did not significantly affect *S. frugiperda* weight gain compared to PBS injections (with the exception of time points 2 h and 6 days; Table 1). Even when higher doses of venom were injected (0.15 and 0.25 w.e.), the weight gain of venom-injected larvae was not significantly different from that of PBS-injected larvae (data not shown). Calyx fluid significantly reduced larval weight gain as early as 24 h post treatment and until the 7th day (Table 1). This indicated that calyx fluid, but not venom, slowed larval weight gain. The weight gain difference between parasitized and calyx fluid-injected *S. frugiperda* could have been due to the presence of the developing parasitoid larvae (hatching normally 48 h post parasitism). Finally, co-injections of venom extract with calyx fluid have the same effect on weight gain as calyx fluid alone, indicating that...
Venom did not have a synergistic effect with calyx fluid on host growth. Thus, consistent with reports for another ichneumonid wasp of the Campopleginae family associated with ichnoviruses, Tranosema rostrale (Doucet and Cusson, 1996), H. didymator venom has no evident effect on S. frugiperda larval development. The calyx fluid of H. didymator appears therefore to be the main factor responsible for the slower weight gain by parasitized larva.

3.3. The H. didymator venom gland proteome

The physiology experiments described above show that H. didymator venom injections affected neither the pre-imaginal survival of H. didymator, nor the S. frugiperda cellular immune response or development. This could be because the venom gland does not produce host-regulation factors, despite its structural characteristics typical of parasitoid venom glands. To assess the functionality of the H. didymator venom gland, we investigated its content using a proteomic approach.

3.3.1. Protein content overview

H. didymator venom was analyzed by SDS-PAGE. It contained numerous proteins ranging in size from 12 to 200 kDa (Fig. 4); although most of the proteins migrated between 20 and 100 kDa, high molecular weight proteins (>170 kDa), characteristic of parasitoids venom extracts (Leluk et al., 1989), were also present. For exhaustive proteomic analysis of the venom extract, the electrophoresis gel was divided into 11 sections. The proteins were extracted from each section and digested with trypsin. The resulting digests were analyzed by LC-MS/MS: 943 H. didymator DNA sequences matching 8946 tryptic peptides were identified (Supplementary data 1 and 4). Note, that these sequences result from the assembly of sequences from EST and 454 databases, and are not necessarily full-length genes. Therefore, there could be redundancy with more than one sequence corresponding to the same H. didymator gene. Consequently, the true number of H. didymator genes corresponding to venom peptides is probably lower than 943.

For an overview of the venom components, each of the 943 sequences was assigned to one of 22 broad functional protein categories based on Blastx searches performed against the NCBI non-redundant database (E-value > e−05) followed by a manually-
complemented automated annotation (Fig. 5). Matches were found for 925 of the 943 sequences, mainly (93%) with hymenopteran proteins (Supplementary data 1). According to this classification, the H. didymator venom extract contained a large number of enzymes, particularly hydrolases, oxidoreductases and transferases. Hydrolases are often reported in venoms (examples and references in Supplementary data 2), including those of ichneumonid wasps. For instance, hydrolase activity has been detected using enzymatic semi-quantitative colorimetric analysis in the venom of the pupal endoparasitoid, Pimpla hypochondriaca (Dani et al., 2005). H. didymator venom contained a few protease inhibitors, a class of proteins also frequent in parasitic and social wasp venoms (Colinet et al., 2009; dos Santos et al., 2010; Yamamoto et al., 2007). The H. didymator venom extract also contained a large number of substrate-specific transporters and storage proteins (e.g. hexamerin, apolipoporphins found in the high molecular weight section of the gel, Fig. 4), proteins that are not commonly found in venoms. However, as for other abundant classes such as mitochondrial, ribosomal, chaperone and structural proteins, and proteins involved in cell trafficking (Fig. 5), it is unclear whether they are indeed true venom components.

This global analysis of the venom content revealed the presence of two categories of proteins which we examined in more detail: (i) a set of proteins not previously described in parasitoid venoms that were encoded by genes strongly transcribed in H. didymator venom apparatus and (ii) a set of proteins similar to proteins found in the venom of other parasitoid species that have host regulatory functions.

3.3.2. Newly discovered H. didymator venom proteins

Among the DNA sequences matching peptides described by the LC–MS/MS analyses, 17% had no similarity with other proteins in generalist databases (Fig. 5). In particular, five sequences corresponded to transcripts that were abundant in the venom EST library (2588 sequences of the 2994 of the EST library, 86%).

3.3.2.1. Gene sequence analyses. Sequencing following PCR amplification of genomic DNA identified these ESTs as corresponding to four distinct genes: three belong to a multigenic family named Hd-VenA; the fourth gene was named Hd-VenB.

The three members of the Hd-VenA multigenic family were named Hd-VenA1 (GenBank accession # BankIt1561820), Hd-VenA2...
The coding region of the *Hd-VenB* gene was 1194 bp long and contained three introns in its 5'-region, as determined by comparing genomic and EST sequences (Fig. 7 A).

### 3.3.2.2. Transcription profiles

The available EST sequences indicate that there is alternative splicing for *Hd-VenA3*, leading to the production of two mRNAs: *Hd-VenA3a* and *Hd-VenA3b* (Fig. 6B). RT-PCR experiments indicated, in accordance with the large number of ESTs (1713, 413 and 331 for *Hd-VenA1*, 2 and 3 respectively), that all *Hd-VenA* transcripts were highly and specifically expressed in the venom apparatus, although a weak *Hd-VenA2* signal was also detected in ovarian tissue (Fig. 6C).

Similarly, the large number of related sequences in the *H. didymator* venom EST library (131 ESTs) indicates that the *Hd-VenB* gene is strongly transcribed in the venom apparatus. However, the specificity of its expression in the venom apparatus could not be clearly established: RT-PCR experiments gave numerous amplification products of varying size both in the venom apparatus.
and in head and thorax (Fig. 7C). Further work is required to elucidate this presence of diverse amplification products.

### 3.3.2.3. Characteristics of Hd-VenA and B proteins

In agreement with the predicted presence of a peptide signal (Figs. 6D and 7D), mass spectrometry analysis detected the corresponding proteins in the venom extract (Supplementary data 1). Hd-VenA are proteins of predicted low molecular weight, 8–18 kDa, consistent with the sections of the SDS-PAGE gel from which they were identified (Fig. 4). The predicted Hd-VenB protein is 310 amino-acids long (34 kDa), in agreement with the detection of most of the peptides corresponding to the protein in Section 4 of the SDS-PAGE gel (Fig. 4). The protein sequence contains a putative signal peptide, and 10 direct repeats of 20 amino acids, as predicted by RADAR software (http://www.ebi.ac.uk/Tools/Radar) (Fig. 7D). None of the proteins displayed a domain conserved in sequences present in generalist databases.

Although they encode proteins predicted to be secreted and found in *H. didymator* venom extract, only a small number of peptides (4–18 peptides) matched with these proteins. In addition, no major bands in the molecular weight range corresponding to these proteins were observed in the electrophoretic profile of the venom (Fig. 4). Therefore, Hd-VenA and Hd-VenB may not be abundant in *H. didymator* venom. On the other hand, they may be unstable or be processed/degraded very rapidly and thus difficult to detect using the above techniques.

### 3.3.2.4. Proteins conserved in Campopleginae wasps

Although Hd-VenA and Hd-VenB proteins have not been previously reported to NCBI/EMBL databases, sequences encoding similar proteins were found in EST libraries from two Campopleginae species, *C. sonorensis* (whole body mixed male and female EST library) and *Venturia canescens* (venom gland EST library, private library).

Alignment of *H. didymator* Hd-VenA protein sequences show 30–45% amino acid sequence identity with translated *C. sonorensis* (CsEST1, LIBEST_026437) and *V. canescens* (VcEST1a and VcEST1b, GenBank accession # KC012591 and KC012592 respectively) EST sequences. Two domains were found to be conserved in all Hd-VenA proteins and also CsEST1 and VcEST1a proteins: a "FGIFx-inv-AxVFAxxxxxxxEEAE" motif in the N-terminal region, and a "RLGRSADMPSMKxxEMIKAR" motif in the C-terminal region (Fig. 6D).

Like Hd-VenA genes, Hd-VenB-related sequence was identified in the venom EST library from *V. canescens* (VcEST2, GenBank accession # KCO12593), suggesting that this gene is also conserved in Campopleginae species and probably transcribed in the venom apparatus (97 of 3085 ESTs in the *V. canescens* library). The *V. canescens* translated sequence showed 53% amino acid identity
with the *H. didymator* protein. However, VcEST2 contained only four of the ten repeats present in Hd-VenB (Fig. 7D).

Thus these genes appear to be conserved among Campopleginae wasps associated either with PDVs or VLPs, and are expressed in the venom apparatus of these phylogenetically closely related species. The presence of the gene products in *C. sonorensis* and *V. canescens* venom extracts has not yet been demonstrated, but at least for *V. canescent*, the genes are strongly transcribed in the venom gland (1078 out of 3085 ESTs for VcEST1a and b). The function of these proteins in the venom apparatus or in the host/parasitoid interaction is unknown.

### 3.3.3. Proteins previously described in the venom of other parasitoid species

Some of the 925 *H. didymator* sequences identified by the proteomic analysis (Table 2) share similarity with proteins identified in the venom of other parasitoid species, including some implicated in altering the host phenotype. We examined this group to determine whether they represent functional venom proteins.

#### 3.3.3.1. Potentially functional hydrolases

Although their function in host/parasitoid interactions is largely uncharacterized, hydrolases are common venom components in many organisms (Vincent et al., 2010). *H. didymator* venom extract contained proteins (Hd-Ven1 and Hd-Ven2) similar to disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) from the repolyspin family, and proteins (Hd-Ven21 and Hd-Ven22) similar to dipeptidyl peptidase IV (DDPPIV). Both corresponded to a large number of peptides identified in the proteomic analysis (Table 2), suggesting they were abundant in the *H. didymator* venom.

Metalloproteases of the repolyspin family have been found in many parasitoid venoms (Table 2) and one acts as a host development alteration factor (EpMP3) in the interaction between *E. pennicornis* and its host *L. olaraca* (Price et al., 2009). Venomous ADAMTS proteins are also commonly found in snakes or in social stinging wasps where they are major active components responsible for tissue damage (degradation of the extracellular matrix, bleeding or inflammation reaction) (de Graaf et al., 2010a; dos Santos et al., 2010). *H. didymator* Hd-Ven1 and Hd-Ven2 proteins showed low similarity to *Nasonia vitripennis* ADAMTS-16 (respectively 30% and 23% identity; Supplementary data 5, Fig. S1). It is not known whether these sequences correspond to a single gene, because they were not complete and correspond to the N-ter (Hd-Ven1) and the C-ter (Hd-Ven2) parts, respectively, of the *N. vitripennis* sequence (Supplementary data 5, Fig. S1). The *H. didymator* repolyspin(s)-like protein migrated in the section 5 of the SDS-PAGE (Fig. 4), deduced from the number of corresponding tryptic peptides, and this is coherent with the reported size of the *P. hypochondriaca* (39.9 kDa) repolyspin (Parkinson et al., 2002a). The available *H. didymator* sequences lacked the N-terminal region so we do not know whether it has a signal peptide and cannot therefore predict whether it is secreted. However, Hd-Ven2 contains the conserved catalytic domain HEXXHXUGUXH suggesting that it may be a functional zinc metallopeptidase (Supplementary data 5, Fig. S1).

DDPPIVs are membrane-anchored enzymes, sometimes associated with exosome membranes, and which cleave N-terminal dipeptides. They are found in the venom of snakes and stinging wasps and were recently reported in *N. vitripennis* (Table 2). Their function in venom remains unclear and may be limited to activation or inactivation of biomolecules within the venom reservoir (Mentlein, 1980) (Ogawa et al., 2008). For instance, in *Apis mellifera*, a membrane-anchored DDPIV activates pro-melittin into highly toxic melittin in the venom reservoir (Krell et al., 1980). DDPPIVs may thus be, as suggested by (de Graaf et al., 2010a), “venom trace elements”. The *H. didymator* Hd-Ven21 and Hd-Ven22 were similar to DPPIV from the venoms of *N. vitripennis* (26% and 49% identity, respectively) and *Vespa basalis* (34% and 60% identity, respectively) (Supplementary data 5, Fig. S2). Hd-Ven21 covered the N-terminal region of the enzyme and Hd-Ven22 the C-terminal region making impossible to determine how many genes code for DDPIV in the *H. didymator* venom gland. The corresponding proteins were recovered from section 8 of the SDS-PAGE (around 80 kDa, Fig. 4), consistent with the sizes of other venom DDPPIVs (86.8 and 88 kDa for venom DDPIV isofom 1 of *N. vitripennis* and *V. basalis* respectively).

Although less abundant in *H. didymator* venom, as indicated by the number of corresponding peptides, one sequence, Hd-Ven43, shared 50% and 45% identity with serine proteases in the venoms of the stinging wasps *Apis florea* and *Bombus ardens*, respectively (Supplementary data 5, Fig. S3). The *Bombus ardens* enzyme is a trypsin-like CLIP-domain serine protease, a family involved both in the activation of the Toll pathway that regulates dorso-ventral axis formation and innate immunity, as well as in the PO signaling pathway (Jang et al., 2008). The Hd-Ven43 sequence also shared 27% identity with *Cotesia rubecula* Vn-50, a serine protease homolog which suppresses the humoral response in the host *Pieris rapae* by competing with the host serine protease involved in the phenoloxidase (PO) activation cascade (Agari, 2012). The *H. didymator* sequence contains part of the catalytic triad suggesting that it may be an active serine protease (Supplementary data 5, Fig. S3).

A number of esterases and glycosylases were also identified in *H. didymator* venom (Fig. 5). Proteomic analyses identified three sequences, Hd-Ven73, Hd-Ven74 and Hd-Ven75, with similarities with the venom carboxylesterase-6 described in *N. vitripennis* (40, 47 and 73% identity, respectively) (Supplementary data 5, Fig. S4). All *H. didymator* carboxylesterase-like sequences contain the catalytic triad (residues S–E–H) characteristic of the esterase-lipase family, suggesting that the enzymes are functional. Hd-Ven83 shows similarities with a hyaluronidase in the venom of the solitary wasp *Anoplius samariensis* (56% similarity) (Supplementary data 5, Fig. S5) and was found in section 5 of the SDS-PAGE (Fig. 4). Hyaluronidases are enzymes that catalyze the hydrolysis of hyaluronic acid and other constituents of the extracellular matrix of vertebrates. They are common in the venoms of snakes (Kemparaju and Girish, 2006), honeybees, wasps and hornets (Schmidt et al., 1986). In parasitoids, hyaluronidase activity has only previously been described in the venom of *Eupelmus orientalis*, an ectoparasitoid of bruchid insects (Doury et al., 1997). Otherwise, an EST sequence coding for a partial hyaluronidase lacking a signal peptide was reported in *C. inanitus* (Vincent et al., 2010). The *H. didymator* sequence we report covers the N-terminal region of the protein and contains a signal peptide (Supplementary data 5, Fig. S5) suggesting that this endoparasitoid enzyme is secreted, like classical wasp venom hyaluronidases.

This analysis of the sequences available suggests that these various enzymes found in *H. didymator* venom extract may be functional hydrolases that could potentially be injected into the host at oviposition and act on the physiology of the host caterpillar.

#### 3.3.3.2. Potentially active serine proteases inhibitors

*H. didymator* venom contained serine protease inhibitors (serpins). Their presumed role in venom is to interact with one or several serine proteases that activate the PO cascade: they thereby may inhibit PO activation, as demonstrated for a serpin (LbSPNy) which is one of the most abundant proteins in the venom of a strain of the figtitid endoparasitoid *Leptopilina boulardi* (Colinet et al., 2009). Peptides from *H. didymator* venom matched a number of sequences displaying similarities with...
Table 2
List of selected proteins identified by nano-LC-MS/MS in *H. didymator* venom extract that share similarities with proteins reported in venoms of others organisms. Predicted proteins are classified according to the nomenclature defined in Fig. 5. For each sequence, the amino acid length, prediction of any signal peptide (using SignalP4 software), the total number of peptides, the number of unique peptides, the peptide redundancy (total/unique), the gel section from which majority of the peptides were obtained and the number of *H. didymator*-related venom EST sequences are indicated. Species from which venom components are reported are: *Apis florea* (Af); *Apis mellifera* (As); *Asobara japonica* (Aj); *Bracon hebetor* (Bh); *Cyphononyx dorsalis* (Cd); *Chelonus inanitus* (Ci); *Cotesia rubecula* (Cr); *Eulophus pennicornis* (Ep1); *Eumenes pomiformis* (Ep2); *Eupelmus orentalis* (Ep2); *Leptopilina boulardi* (Eo); *Lepidoptera* (Lb); *Microctonus hyperodae* (Mh); *Orancistrocerus drewseni* (Nv); *Pimpla hypochondriaca* (Od); *Polybia paulista* (Ph); *Pteromalus puparum* (Pp1); *Pteromalus puparum* (Pp2); and *Vespid basalis* (Vb). Cc, Ci and Cr are braconids which have associated polydnavirus; Ma and Mh carry virus-like particles. Asterisks (*) indicate that only the transcript has been identified in a venom gland EST library; double asterisks (**) indicate that only the activity has been detected in crude venom by enzymatic tests or antibody-inhibition experiments; symbols (x) indicate that data originates from automatic genome annotation. (1) Abt and Rivers, 2007. (2) Asgari et al., 2003b. (3) Baek and Lee, 2010. (4) Birrell et al., 2007. (5) Cardoso et al., 2010. (6) Carrijo-Carvalho and Chudzinski-Tavassi, 2007. (7) Colinet et al., 2009. (8) Colinet et al., 2011. (9) Crawford et al., 2010a. (10) Crawford et al., 2010b. (11) de Graaf et al., 2010a. (12) de Graaf et al., 2010b. (13) dos Santos et al., 2010. (14) dos Santos et al., 2011. (15) Doury et al., 1997. (16) Ersfeld et al., 2006. (17) Lee et al., 2007. (18) Mentielen et al., 1999. (19) Padavattan et al., 2008. (20) Parkinson et al., 2001. (21) Parkinson et al., 2002a. (22) Peire et al., 2005. (23) Peire et al., 2004. (24) Peire et al., 2008. (25) Price et al., 2009. (26) Rivers et al., 2009. (27) Safavi-Hemami et al., 2010. (28) Schevchenko et al., 2005. (29) Schmidt et al., 1986. (30) Undheim and King, 2011. (31) Veiga et al., 2005. (32) Vincent et al., 2010. (33) Wright et al., 1973. (34) Yamamoto et al., 2007. (35) Zhang et al., 2006. (36) Zhu et al., 2010.

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References

serpins, including an Antithrombin-III from the ant Camponotus floridanus (Hd-Ven383) and a leukocyte elastase inhibitor-like protein from Bombus impatiens (Hd-Ven384) (Supplementary data 1). However, only one of these serpin-like sequences, Hd-Ven390, contains a predicted putative functional reactive center loop (RCL). This protein was well represented in H. didymator venom extract (Table 2) and shares similarities with a neuroserpin-like protein in the bee Microctonus rotundata and with the LbSPN protein (50% and 30% identity, respectively) (Supplementary data 5, Fig. S6). The deduced size of Hd-Ven390 was 45 kDa (42 kDa after peptide signal cleavage), consistent with the recovery of most of the tryptic peptides from the section 5 of the SDS-PAGE gel (Fig. 4). Preliminary data suggest that H. didymator venom extract does not prevent PO activation (data not shown), so role, if any, remains unclear. Hd-Ven390 may indeed be an active secreted venom serpin but which acts inside the venom apparatus rather than as a true virulence factor, as suggested for a venom serpin from the social wasp Polybia paulista (dos Santos et al., 2010).

3.3.3.3. Other enzymes. Superoxide dismutase (SOD) is an oxidoreductase found in many wasp venoms (dos Santos et al., 2010; Peiren et al., 2008). In many cases, an intracellular form of the SOD is detected, suggesting that the protein may be a cellular contaminant or act on the maintenance of the venom apparatus (Peiren et al., 2008). One exception is the extracellular SOD abundant in the venom gland of the parasitic wasp L. boulardi that inhibits PO activity in vitro (Colinet et al., 2011). The two SODs identified in H. didymator venom (Hd-Ven218 and Hd-Ven219; Table 2) display substantial similarities (more than 80% identity) with intracellular SODs of Asobara tabida and L. boulardi and weaker similarities (33 and 50%, respectively) with L. boulardi extracellular SOD (Supplementary data 5, Fig S7). The H. didymator sequences do not contain predicted signal peptides and thus most probably correspond to intracellular SODs.

H. didymator venom also contains a proPO, the inactivezymogen form of PO (Hd-Ven204; Table 2). A PO containing a peptide signal allowing secretion of the enzyme has been described in P. hypochondriaca venom (Parkinson et al., 2001). No peptide signal was predicted for the H. didymator protein which shares some similarity (45% identity) with the secreted P. hypochondriaca PO and more similarity (71% identity) with A. mellifera proPO (Supplementary data 5, Fig. S8). Hd-Ven204 is therefore probably a classical cellular enzyme released by regulated exocytosis as described for PO found in hemocytes of many arthropods (Lai et al., 2002).

Another abundant enzyme in H. didymator venom, as evaluated from the number of peptides, was an arginine kinase highly similar (>70% identity) to other hymenopteran sequences (Table 2; Supplementary data 5, Fig. S9). Arginine kinase has been reported in the venom of several hymenopteran insects but there is only one report of a paralytic effect of this type of enzyme: a truncated (25 kDa) form in the spider-hunting wasp Cyphonexy dorsalis (Yamamoto et al., 2007). The arginine kinase identified in H. didymator was found in section 4 of the SDS-PAGE (~30–40 kDa, Fig. 4) and was thus most probably a full-length enzyme and likely to be intracellular.

Therefore, according to the analysis of the available sequences, the three enzymes described above appear to be intracellular enzymes and probably are not injected into the insect host.

3.3.3.4. Non enzymatic proteins. Diverse non enzymatic proteins, as found in the venom of various parasitic wasps, were also identified in H. didymator venom. These proteins include immune-related proteins, such as calreticulin and a C1q-like venom protein, a C-type lectin and venom allergen proteins (Table 2).

Calreticulin (CRT) is a multifunctional Ca²⁺-binding chaperone protein found in the venom fluid of the parasitic wasps C. rubecula, P. puparum and N. vitripennis (Table 2). In C. rubecula, CRT prevents encapsulation in vitro by inhibiting hemocyte spreading behavior, although the mechanism is still unclear (Zhang et al., 2006). One study on ichneumonidae P. hypochondriaca venom demonstrated a hemocyte anti-aggregation effect of the venom CRT (Rivers et al., 2009). A C1-like protein, Hd-Ven392, highly similar (more than 70% identity) to other parasitic wasp CRTs was found in H. didymator venom (Supplementary data 5, Fig. S10). The number of corresponding peptides was low (Table 2) and although this CRT appears to be secreted and functional, the quantity present in H. didymator venom may be insufficient to cause a significant effect once injected into the host caterpillar.

The H. didymator venom extract also contained a protein with similarities to the Tumor Necrosis Factor superfamily member C1q (Hd-Ven186), a subunit of the C1 enzyme complex that activates serum complement. A C1q-like venom protein precursor has been identified in N. vitripennis and A. mellifera (61 and 57% identity, respectively, with Hd-Ven816; Supplementary data 5, Fig. S11) (de Graaf et al., 2010b). In N. vitripennis, the protein is also present in a number of tissues other than the venom gland and it was suggested that it is not a true virulence factor (de Graaf et al., 2010a). CRT blocks the interaction between C1q and immunoglobulins in humans, so the two proteins may interact in the venom gland where they are both found, such as suggested for N. vitripennis (Danneels et al., 2010).

H. didymator venom also included a protein with a C-type lectin (CTL) domain (Hd-Ven831). Venomous CTLs have only been described in braconid wasps carrying PDV as C. inanitus (Vincent et al., 2010) where the protein is believed to play a role in immune-suppression, in the same way as proteins with a CTL domain encoded by the PDV associated with the braconid Cotesia plutellae (Lee et al., 2008). The H. didymator sequence is dissimilar to the C. inanitus venom CTL protein (29% identity) (Supplementary data 5, Fig. S12), but resembles other non-venom specific secreted hymenopteran CTLs (89% identity with the CTL protein from Acromyrmex echinatior).

Finally an abundant protein in H. didymator venom, Hd-Ven850, shares similarities with venom allergen-3 (or Antigen 5), a protein of the Antigen 5 family (Ag5) with a cysteine-rich secretory domain. This type of protein, commonly found in Hymenoptera venoms, has been described in ants (Hoffman, 2006) and in several parasitic wasps (Table 2). Antigen 5-like proteins generally have a signal peptide for secretion (Padavattan et al., 2008) but their biological function is still not known. The available deduced H. didymator sequence (80 aa) encompasses the C-terminal region of the protein and shares 25% identity with other allergen-3 sequences (Supplementary data 5, Fig. S13). Its predicted size of 20 kDa most probably corresponds to the major band in Section 3 of the SDS-PAGE (Fig. 4). In the allergen category, we also identified an icarapin-like peptide, Hd-Ven841, a novel IgE-binding bee venom protein of unknown biological function (Peiren et al., 2006). The available sequence has a signal peptide, typical of secreted proteins (Supplementary data 5, Fig. S14).

4. Conclusion

This first exhaustive analysis of a Campopleginae parasitic wasp venom proteome, that of H. didymator, reveals the presence of several components related to venomous proteins. This is in apparent contrast with the observation that injection of venom extract does not have any detectable effect on development or cellular immunity of the host, S. frugiperda. There are various possible reasons for the venom having no apparent effect on host
physiology. Firstly, many proteins that were identified in *H. didymator* venom are not commonly major components of active venoms of koinobiont species, although they are often reported in these species. The toxicity of many of these molecules, such as metalloproteases, arginine kinase or hyaluronidase, has only been demonstrated in stinging wasps, and most of them are allergen like molecules. The early work of V. canescens, a species devoid of PDVs but associated with VLPs; for this species, there is also no clear evidence of an effect of the venom on host physiology. Only a transient paralysis following venom injection has been reported (Piek, 1986). Similarly, for *C. sonorensis*, which is associated with PDV and also contains homologos, no effect of the venom on parasitism success was reported (Webb and Luckhart, 1994). Further investigation is needed to understand functions of these proteins, which may be in the venom gland (e.g. venom storage or tissue protection) rather than during parasitism.

Our study strongly suggests that there are substantial differences in the evolution of venom components between the two families associated with PDVs, the Braconids and the Ichneumonids. In *H. didymator*, calyx fluid alone was sufficient to reproduce the parasitized host phenotype, which is not the case for braconids, where the venom synergizes with PDV (Asgari, 2012). Preliminary analyses also indicated that *H. didymator* venom had no significant effect on the transcription levels of four HdHV (P30, M24, VanK1803 and VanKCR1) genes (data not shown). Indeed, the extensive analysis of *H. didymator* venom did not identify components related to proteins demonstrated to synergize with either the effects or expression of PDV in Braconidae venom. Most Campopleginae species studied to date are endoparasitoids that rely on PDVs or VLPs produced in the ovarian tissues to manipulate host physiology. In these species, "viral" host regulatory factors may have been acquired during evolution before specialization of the venom to have host regulatory functions or viral acquisition may have been followed by the loss of any such function of the venom. Braconids domesticated a virus independently, and consequently PDV acquisition appears to have led to a different evolution of the relationship between the PDV and the venom, with venom molecules required for the success of parasitism. The reasons why the venom gland in Campopleginae species retains the capacity to produce a large range of molecules remains to be elucidated. It is possible that Campopleginae venom simply corresponds to a phylogenetic signal and no longer has a role, but it may still have an ancestral role in ovipositor lubrication or may have acquired new functions that have not been identified yet.

Many aspects of the evolution of the nature and function of venoms in parasitic wasps, and the consequences of the acquisition of symbiotic PDV or VLP in ichneumonid wasps remain to be elucidated. Analysis of the venom proteomes in a range of species in different branches of the phylogenetic tree of the Ichneumonidae would be informative. Ichneumonids display a great variety of parasitoid lifestyles and there may have been multiple shift events between ecto- and endoparasitism (Gauld, 1988). Investigation of the venoms of koinobiont species from the Cremastinae, Anomaloneae and Ophioninae sub-families of Ichneumonids, which may not carry PDV or VLP, may identify venomous factors required for parasitism success, and contribute to our understanding of Campopleginae venom evolution.

The evolution of venoms in other groups, such as reptiles whose venoms only have a function in predation, is better characterized (Wuster, 2010) than that of venoms in Hymenoptera, and more particularly in parasitic wasps, with their enormous diversity of life cycle strategies. Currently, only a few venomous proteins have been demonstrated to play a role in the host—parasitoid interaction. Furthermore, they generally have been described in only a small number of genera, reflecting the limited exploration of parasitoid venom diversity. Following on from the pioneer work based on the study of venom apparatus morphology (Robertson, 1968), and venom protein and enzymatic profiles (Leluk et al., 1989; Schmidt et al., 1986), recent advances in the characterization of hymenoptera venoms should, as illustrated by our study, provide a better insight into venom evolution in the context of the highly diverse life-styles of hymenoptera.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2012.12.010.

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